CORRECTION

Correction: Boat noise interferes with Lusitanian toadfish acoustic communication

Daniel Alves, Manuel Vieira, M. Clara P. Amorim and Paulo J. Fonseca

There were several errors in *J. Exp. Biol.* (2021) **224**, jeb234849 (doi:10.1242/jeb.234849).

Owing to a mistake during production, a number of corrections requested by the author were not made.

These changes include the following explanation added to the 'Sound stimuli' section of the Materials and Methods for the use of 1 m rather than 0.1 m as a distance for measuring boatwhistles:

We assumed a BW amplitude of 140 dB (re. 1 µPa) at 0.1 m as in Alves et al. (2016), which corresponds to a BW of a toadfish of c. 25 cm SL (Vasconcelos and Ladich, 2008). The maximum amplitude playback used in the experiments was adjusted to 130 dB (re. 1 µPa) corresponding to the estimated amplitude at 1 m from a toadfish nest (Alves et al., 2016). Note that BW amplitude changes with male size (Vasconcelos and Ladich, 2008) and attenuation is highly dependent on water level (Alves et al., 2016).

Abstract, line 15, now reads 2.0–2.5 m for the motorboat, rather than 1.7–2.5 m.

Results, second paragraph, line 5, active space for BW1 was changed to 6.4 to 2.0 m, instead of 6.4 to 1.7 m.

Results, second paragraph, line 11, significance level was changed to $P=0.8$, rather than $P=0.9$.

In Figs 2, 3, 7 and 8, BW recording distance was changed to 1.0 m, rather than 0.1 m.

In Table 1, small boat detection distance for BW1 was changed to 1.0 m (rather than 0.1 m) and mean value to 2.0±0.8 m (rather than 1.7±1.2 m).

These changes do not impact the conclusions of the paper.

All versions of the article have been updated. We apologise to the authors and readers for this error and any inconvenience it may have caused.

RESEARCH ARTICLE

Boat noise interferes with Lusitanian toadfish acoustic communication

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ABSTRACT

Anthropogenic noise is considered a major underwater pollutant as increasing ocean background noise due to human activities is impacting aquatic organisms. One of the most prevalent anthropogenic sounds is boat noise. Although motorboat traffic has increased in the past few decades, its impact on the communication of fish is still poorly known. The highly vocal Lusitanian toadfish (*Halobatrachus didactylus*) is an excellent model to test the impact of this anthropogenic stressor as it relies on acoustic communication to attract mates. Here, we performed two experiments to test the impact of boat noise on the acoustic communication of the Lusitanian toadfish. Using the auditory evoked potential (AEP) technique, we first compared the maximum distance a fish can perceive a boatwhistle (BW), the mate attraction acoustic signal, before and after embedding it in boat noise. Noises from a small motorboat and from a ferryboat reduced the active space from a control value of 6.4–10.4 m to 2.0–2.5 m and 6.3–6.7 m, respectively. In the second experiment we monitored the acoustic behaviour of breeding males exposed to boat noise playbacks and we observed an increase in the inter-onset interval of BWs and a disruption of the usual vocal interactions between singing males. These results demonstrate that boat noise can severely reduce the acoustic active space and affect the chorusing behaviour in this species, which may have consequences in breeding success for individuals and could thus affect fitness.

KEY WORDS: Boatwhistle, Fish, Communication range, AEP technique, Anthropogenic noise, Vocal patterns

INTRODUCTION

Acoustic communication during social interactions is widespread in animals (Bradbury and Vehrencamp, 1998), including teleost fish, which are considered the largest group of vocal vertebrates (Ladich, 2004). In these animals, sounds are produced in contexts such as agonistic interactions, competitive feeding, disturbance, advertisement, courtship or spawning (Bradbury and Vehrencamp, 1998). Taking these last three contexts together, acoustic communication may have a significant impact in the reproductive success of animals (e.g. Vasconcelos et al., 2012).

The correct interpretation of the information present in a sound signal requires that it propagates without severe distortion of its informative features and that the receiver correctly extracts the information (Bradbury and Vehrencamp, 1998). Sounds attenuate with distance, which imposes a limit to the range at which an acoustic signal can be correctly perceived. This distance is known as the active space (Clark et al., 2009) and is very important for successful acoustic communication. Despite its crucial role for successful communication, the active space of acoustic signals has received little attention in fish and has so far been estimated mostly in shallow water conditions and only in a small number of fish, namely *Opsanus tau* (Fine and Lenhardt, 1983), *Pomacentrus partitus* (Myrberg et al., 1986), *Dascyllus albisella* (Mann and Lobel, 1997), *Padogobius martensii* (Lugli and Fine, 2003), *Gobius nigricans* (Lugli and Fine, 2003), *Pogonias cromis* (Locascio and Mann, 2011), *Ictalurus furcatus* (Ghahramani et al., 2014), *Cyprinella venusta* (Holt and Johnston, 2015), *Pempheris adspersa* (Radford et al., 2015), *Halobatrachus didactylus* (Alves et al., 2016), *Gadus morhua* and *Melanogrammus aeglefinus* (Stanley et al., 2017). In shallow waters, fish sounds, which typically have most of their energy in frequencies below a few hundred Hertz, attenuate to background levels within a short distance from the sender. This happens because when the wavelength of the sound is longer than the water column (e.g. $\lambda \approx 15$ m for a 100 Hz sound), the sound is strongly attenuated (Bass and Clark, 2003; Mann, 2006). Estimated active space varies in different species from a few centimetres to tens of metres depending on signal amplitude, water depth and substrate type (Amorim et al., 2015).

While acoustic signals and auditory mechanisms have evolved under natural noise (Tuset et al., 2016), increased underwater noise due to human activities (anthropogenic noise) is likely to impose new constraints on communication. This increased noise level can mask fish sounds by causing a reduction in their active space and impairing the detection of key signal features such as amplitude modulation or frequency content (Ladich, 2013). Anthropogenic noise produced by activities such as boating, pile driving or seismic surveys, has been recognized as an underwater global pollutant and is a growing international concern (Slabbekoorn et al., 2010; Popper and Hawkins, 2016). The detrimental effects of man-made noise ranges from mild effects, such as behavioural avoidance or temporary threshold shifts, to effects as severe as direct mortality (Popper and Hastings, 2009). There is, however, little information on how fish acoustic active space is affected by anthropogenic noise, and controlled systematic research is needed to understand the extent to which anthropogenic noise affects acoustic communication in fishes (Ladich, 2013; Brumm, 2014; Radford et al., 2014).

The Lusitanian toadfish, *H. didactylus*, is a gregarious vocal species with an unusually rich repertoire for a fish (Amorim et al., 2008) that relies on acoustic communication for mate finding and attraction (Vasconcelos et al., 2012) and for the spacing out of territorial males (Vasconcelos et al., 2010; Conti et al., 2015). The more commonly produced sound – the boatwhistle (BW) – is used both to attract females and repel possible intruders (Vasconcelos

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et al., 2010, 2012; Conti et al., 2015). A reduction in BW active space will likely affect mate detection distance and vocal interactions amongst neighbouring territorial males, with implications for fitness. In previous studies, both active space (Alves et al., 2016) and vocal behaviour patterns (Amorim et al., 2011; Jordão et al., 2012; Vasconcelos et al., 2011; Vieira et al., 2021) have been studied. Alves et al. (2016) showed that in shallow habitats BWs can be perceived up to about 13 m. Lusitanian toadfish adjust the rate of BW emission depending on the acoustic social environment, that is, whether they are calling alone or in a chorus (Amorim et al., 2011). In addition, this species exhibits fine-scale male–male interactions, such as matching a neighbour's calling rate (Jordão et al., 2012) and maintaining call alternation, thus avoiding the vocalizations of their neighbours (Vieira et al., 2021). Here, we aim to estimate to what extent boat noise reduces active space and how it affects vocal behaviour of Lusitanian toadfish breeding territorial males.

MATERIALS AND METHODS
Auditory evoked potential technique
Experimental animals
Lusitanian toadfish [*Halobatrachus didactylus* (Bloch and Schneider 1801)] were collected through trawling in the Tagus estuary (Portugal) by local fishermen, from December 2013 to February 2014 and in August 2016, and transported to the laboratory at the University of Lisbon (Portugal). They were kept in 80 litre stock tanks equipped with protein skimmers and aeration, under a 12 h:12 h light:dark cycle, and fed with shrimp once a week. Water temperature ranged between 15 and 17°C (in a shared species bioterium), falling within natural values (10–24°C; Amorim et al., 2006). We used a total of 37 adult fish (24 males and 13 females; body mass 110–1240 g; standard length 16.3–34.4 cm). Following the experiments, the animals were released back to the wild in the same estuary. All experimental procedures complied with European animal welfare laws, guidelines and policies.

Experimental procedure
The experimental procedure was similar to that used in Alves et al. (2016). Briefly, fish were anaesthetized with ethyl p-aminobenzoate (0.01% m/V, Alfa Aesar, Karlsruhe, Germany) and immobilized with gallamine triethiodide (10-15 µg kg^{-1}, Sigma-Aldrich, St Louis, Missouri, USA). Gallamine triethiodide has been commonly used to paralyze fish during auditory evoked potential (AEP) recordings (for effects of anaesthesia on AEPs, see Cordova and Braun, 2007) and do not appear to produce sensory deficits (Smith and Schauf, 1981; Foutz et al., 1983). Nevertheless, gallamine triethiodide has some inhibitory effect on acetylcholine muscarinic receptors (Clark and Mitchelson, 1976), which can inhibit the release of acetylcholine at the efferent hair cell synapse in mammals and anurans. Since one function of cholinergic efferents is to unmask signals in noise (Tomchik and Lu, 2006), it is possible that the use of this immobilizing agent during AEP recordings could block this unmasking effect and result in an overestimation of the impact of boat noise on auditory processing.

The immobilized test subjects were positioned just below the water surface, with their gills perfused with temperature-controlled saltwater at 21±1°C (Fig. 1). This temperature was chosen because it allowed for comparisons with previous studies of this species (e.g. Vasconcelos et al., 2011; Alves et al., 2016) and since it is a common water temperature during the breeding season, that reaches its peak between June and August (17–22°C; Amorim et al., 2006). A short acclimation period was allowed before the experiments (approximately 30 min).
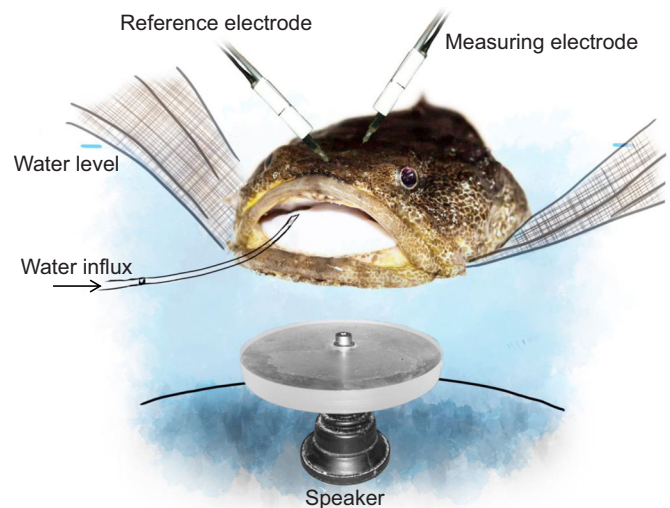

Fig. 1. The experimental procedure for measuring the auditory evoked potentials (AEPs). Test subjects were positioned just below the water surface, with their gills perfused with temperature-controlled saltwater (21±1°C). Acoustic stimuli were fed via an Edirol UA-25EX, amplified, and delivered through an underwater sound generating device.

Acoustic stimuli (see below) were produced on a PC, fed via an Edirol UA-25EX (Roland Corporation, Tokyo, Japan) to an amplifier, and delivered through an underwater sound generating device (described in Vasconcelos et al., 2011 and Alves et al., 2016). The sound generating device was composed of an immersed plexiglass disc driven by a mechanical wave driver (SF9324, PASCO, Roseville, CA, USA) kept below the experimental tank. The disc was attached to the wave driver by a stainless-steel rod which crossed the tank bottom through a water restraining flexible device.

AEPs are summed potentials of the electrical nervous system activity induced by an auditory stimulus. To record the AEPs, a measuring electrode was pressed against the skin of the fish's head directly above the hindbrain, while the reference electrode was positioned between the nares (Vasconcelos and Ladich, 2008; see Fig. 1). The electrical potentials detected by the electrodes were amplified (Grass CP511, Grass Instruments, USA, gain 20,000×, high-pass 10 Hz, low-pass 1000 Hz), digitized (Edirol UA25-EX, Roland Corporation, Tokyo, Japan: 48 kHz, 16 bit) and recorded to a PC running Adobe Audition 3.0 (Adobe Systems Inc., CA, USA). Simultaneously, the second channel of the Edirol recorded square waves synchronized with the beginning of the acoustic stimuli, to be used as trigger signals during AEP analysis.

Sound stimuli
Two different BWs produced by breeding territorial males in a natural breeding habitat (Air Force Base no. 6, Montijo, 38°42′ N, 8°58′ W) were selected to represent the natural variability of this sound (Fig. 2). The chosen BWs have a small difference in duration (602 vs 687 ms, less than 100 ms compared to a variability of ~400–1000 ms often found in BWs). Note that BWs are highly stereotyped, at least in time frames of several minutes of calling (Amorim et al., 2011). According to Amorim and Vasconcelos (2008), BWs have a mean duration of 767.2±168.9 ms (mean±s.d.) and a range from 458.0 to 1052.4 ms. BW1 exhibits similar energy at 50–60 Hz, 100–110 Hz and 150–160 Hz, but has ~10 dB more energy around 50–60 Hz than BW2, where this frequency range is also 10 dB below the energy found at 100–110 Hz and 150–160 Hz. Differences in this frequency band were shown to have an impact in communication active space (Alves
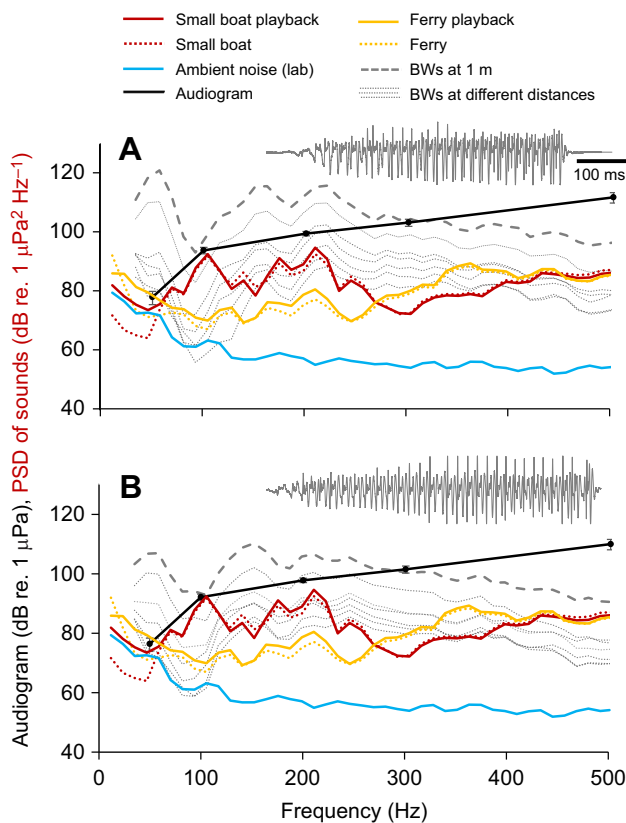
Fig. 2. Power spectra of boat noises and Lusitanian toadfish (*Halobatrachus didactylus*) boatwhistles (BWs) that were combined in the AEP experiment. The grey dashed lines represent the BW spectrogram at different distances: (A) BW1 and (B) BW2. The small fishing motorboat with an outboard engine has more energy at lower frequencies (below 280 Hz) than the ferry and, therefore, has more potential to mask the BWs. Blue line represents the ambient noise. The red and yellow dashed lines represent the adjusted spectra of the original recordings of boat noises. Note that the 1250 ms of boat noise playback used in the experiments were adjusted to 130 dB (re. 1 μPa). Comparison with a baseline audiogram of the Lusitanian toadfish is provided (Vasconcelos et al., 2007). Note, however, that for estimation of masking, critical ratios are needed (Erbe et al., 2016) but are unknown for this species. Spectra settings: sampling frequency, 48 kHz; FFT size, 4096; window type, Hanning; window overlap, 50%. PSD, power spectral density.

et al., 2016) (Fig. 2). This choice was made because dominant frequency was shown to influence the active space of the BWs, while duration had no influence (Alves et al., 2016). We used BW1 and BW2, respectively BW2 and BW3 from Alves et al. (2016), each of which were simultaneously registered with different hydrophones kept at different distances from the sound-producing fish (0.1, 2.5, 5, 7.5, 10, 12.5, 15 m), thus incorporating the effects of attenuation with distance at this breeding habitat. The two BWs were recorded in the same transect with tide levels of 2.25–2.35 m. Since these recordings were made at high tide, the propagation conditions were the best in this habitat (Rogers and Cox, 1988). To evaluate the probable masking caused by boat noise we selected noise from two different boat types common in the area: a small fishing private open deck boat with an outboard engine, recorded 5–10 m from the hydrophone, and a ferryboat that regularly crosses the Tagus river, recorded at about 50 m. These different noises have different frequency components and thus might mask BWs differently (cf. Fig. 2). Note that two sounds recordings do not represent the full variability of each boat

type but gives a snapshot of how spectral content might be responsible for the masking observed.

To simulate a situation where an approaching fish would have to extract information from a BW produced by a territorial male under noise from a passing boat, we mixed the boat noise with the BW recordings at different distances from the calling male obtained by Alves et al. (2016).

We assumed a BW amplitude of 140 dB (re. 1 μPa) at 0.1 m as in Alves et al. (2016), which corresponds to a BW of a toadfish of c. 25 cm SL (Vasconcelos and Ladich, 2008). The maximum amplitude playback used in the experiments was adjusted to 130 dB (re. 1 μPa) corresponding to the estimated amplitude at 1 m from a toadfish nest (Alves et al. 2016). Note that BW amplitude changes with male size (Vasconcelos and Ladich, 2008) and attenuation is highly depend on water level (Alves et al., 2016). Similarly, we then adjusted the amplitude of a 1250 ms playback of boat noise to 130 dB (re. 1 μPa), corresponding to a ferryboat passing ∼50 m away from our study breeding site. The boat noise file at 130 dB was then mixed with the BWs recorded at the different distances (up to 15 m away from the calling male) preserving the decreasing amplitude of the BW on these field recordings. In the playback stimuli the BW started 250 ms after the beginning of the boat noise. These sound stimuli were then used in the AEP experiments (Figs 2 and 3). Each stimulus (1250 ms) was presented 1000 times (2×500 times at opposite polarities), with intervals between presentations equal to 50% of the stimulus duration (625 ms), totalling approximately 4 h 15 min. Each individual was subjected to one combination of BW+boat noise, with the order of the stimuli corresponding to the different distances being randomly selected to minimize possible habituation.

To control for differences in the patterns of particle motion and pressure components of the playback sounds in the AEP setup (Parvulescu, 1967), we compared accelerometer (M20-040, sensitivity 1 Hz–3 kHz, GeoSpectrum Technologies, Dartmouth, Canada) and hydrophone (8104, Brüel and Kjær, Naerum, Denmark; sensitivity −205 dB re. 1 V μPa^{-1}; frequency response from 0.1 Hz to 180 kHz) measurements. The sensors were roughly positioned in the place later occupied by the fish hearing structures, while stepping down (by 6 dB steps) tone playbacks at different frequencies (15, 30, 60, 100, 200, 300, 400, 500, 800 and 1000 Hz). The playback sound pressure amplitudes varied from 130 to 82 dB (re. 1 μPa) (range encompassing the hearing sensibility of this species; Vasconcelos et al., 2007). These measurements showed that pressure and particle acceleration vary approximately in a similar manner in the experimental tank. In fact, in the primary axis of particle motion (the vertical z axis), a 6 dB change in SPL was generally accompanied by a 6 dB change in particle acceleration level (also observed in the same setup with a different accelerometer by Vasconcelos et al., 2011). Regarding the BW and BW+noise stimuli, power spectral density (PSD) plots of sound pressure and particle acceleration components exhibited a very similar energy distribution and a considerable dynamic range (Fig. 3). The pressure amplitude differences between the sounds recorded in the field at the different distances were preserved in the particle acceleration domain. Nevertheless, some differences were noticeable specially between 20 and 150 Hz. Additionally, the acceleration components in the x and y axes had much less energy than the z axis component (10–30 dB difference, data not shown).

Active space estimation

To determine the maximum communication distance, we used the same method as in Alves et al. (2016). In short, BW envelopes were
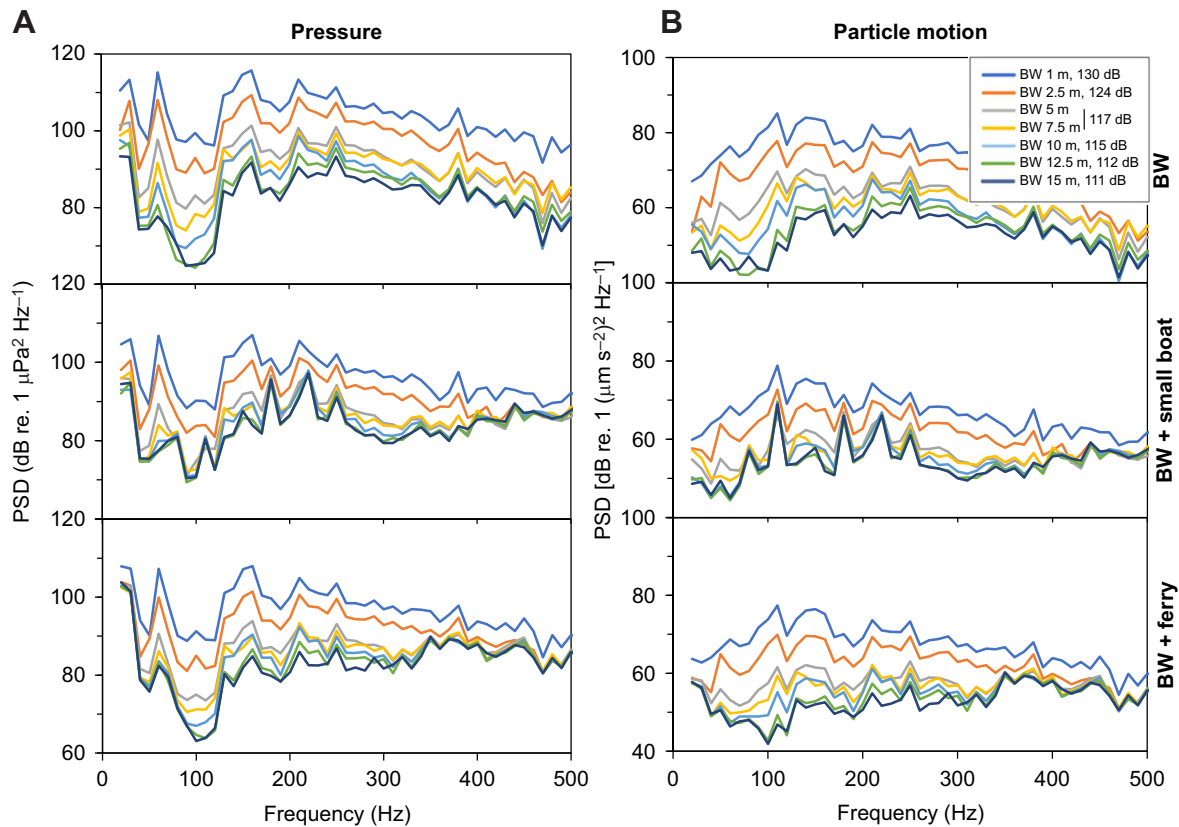
Fig. 3. Power spectral density plots of the playbacks performed in the experimental tank. (A) Sound pressure and (B) particle motion (z-axis) components, of BW2 recorded at different distances without noise, with noise of the small outboard engine motorboat and with the noise of the ferry.

extracted from both the original BW stimulus and the AEP responses (with and without noise) and compared using Pearson's correlations. A threshold was derived from the correlation values calculated between the envelopes of AEP recordings without sound stimulation and the BW envelope (threshold=average+2×s.d., n=24). In experiments where the correlation value was above the threshold value, we considered that the BW was correctly represented in the AEP. The maximum distance where the correlation was above threshold was considered the maximum communication distance. Maximum distance estimations in the presence of boat noise made in this work were compared with those obtained in Alves et al. (2016) with the same BWs and protocol but without boat noise added to the stimulus. This allowed us to estimate communication range distance, i.e. the distance at which a conspecific may extract relevant information from BWs.

Signal averaging was made with custom-made software (P.J.F. and M.V.). The Pearson's correlation analysis was performed with Statistica 12.0 (StatSoft, Inc., USA). To assess differences between AEP responses to the BWs (recorded in the field at the different distances) embedded in noise, a Kruskal–Wallis test was used. This test was selected since homogeneity of variances was not met. *Post hoc* Dunn tests with BH correction were used for pairwise comparisons. These statistical analyses were conducted in R (https://www.r-project.org/).

Fish vocal behaviour patterns
Experimental setup
A field experiment monitoring the vocal behaviour of toadfish territorial breeding males while exposed to boat noise playbacks was set up in a nesting field site (Air Force Base no. 6, see

coordinates above) (Fig. 4). This setup consisted of 12 concrete artificial hemicylindrical nests capped at one end (50 cm long, 30 cm wide and 20 cm maximum height), each one had a custom-made hydrophone placed next to it in mid-lateral position and about 10 cm above the substrate. The hydrophones were connected to a 16-channel stand-alone data logger (Measurement Computing Corporation LGR-5325, Norton, VA, USA, 16 bits, 4 kHz sampling rate). The 12 nests were placed 2 m apart in two rows, at the lower level of spring tides, allowing nests to be permanently underwater for ~10 days in a fortnight, similarly to previous studies (e.g. Jordão et al., 2012; Amorim et al., 2016). Boat noise was played back through 3 UW-30 underwater loudspeakers (ElectroVoice, Burnsville, Minnesota, USA), placed in the middle of the nest rows, ~10 cm from the substrate and facing up, separated by ~3.5 m. Each speaker was fed by an amplifier (Sony XM-N1004, Tokyo, Japan) connected to a mp4 (A730 Music Player, HOTT, Shenzen, China) that produced the sound stimuli. While these loudspeakers have a poor performance and lose power at frequencies below 100 Hz, previous studies have used them with success (e.g. Jordão et al., 2012; Amorim et al., 2016).

We used 7 days of round-the-clock recordings made in June 2019. During the recording period the nests were permanently submersed, the water column varying between ~0.6 and 2.9 m. Temperature recorded at a pier close by (Air Force Base no. 6 Pier) ranged from ~19 to 22°C. Male subjects occupied the nests spontaneously and could move freely.

Playback sound stimuli
Noise playback mimicked the passage of 10 ferries and 4 small boats per hour, approximating traffic that fish can experience in the Tagus
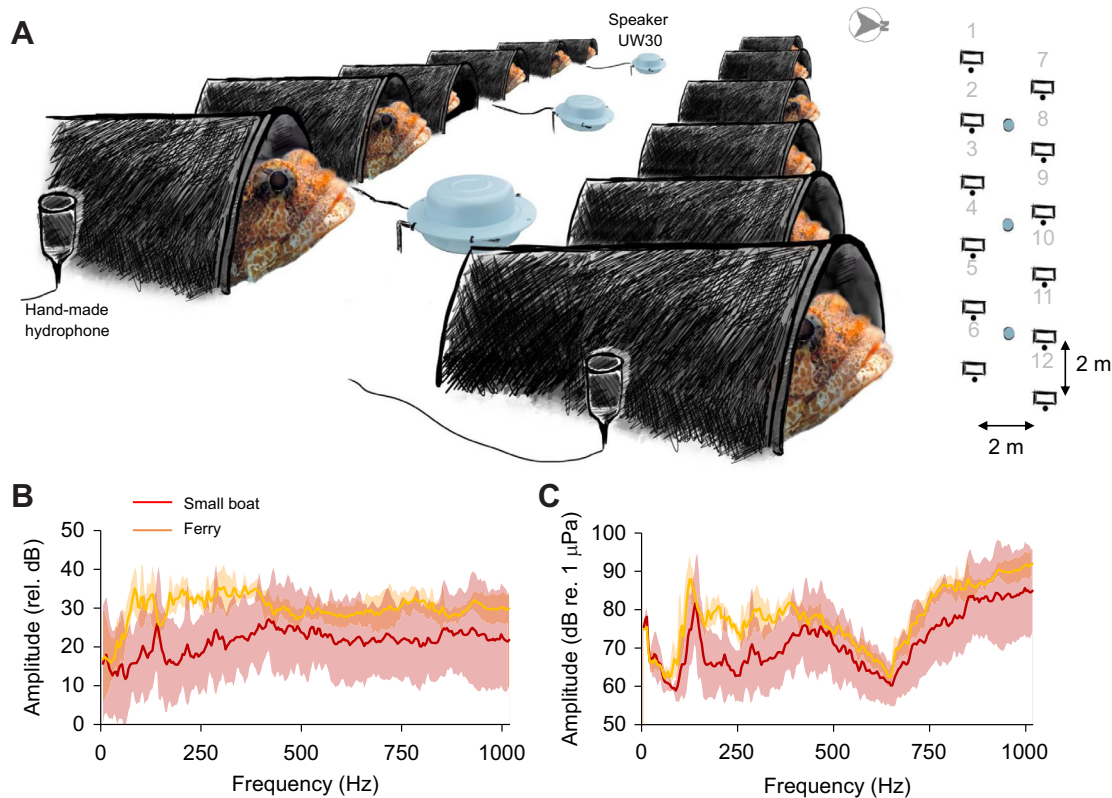
Fig. 4. Experimental setup to monitor fish vocal behaviour patterns in the playback experiment. (A) Schematic depicting the experimental field set up for the assessment of fish vocal exposure in the presence of boat noise playback; we used 12 concrete nests, 3 underwater speakers and 12 custom-made hydrophones; each Lusitanian toadfish male spontaneously occupied an artificial hemicylinder concrete shelter, which were placed 2 m apart. (B) Mean power spectra of different ferry and small boat sounds recorded at Air Force Base no. 6 pier and used to create the playbacks for the field experiments (s.d. shown by shaded colours). (C) Mean power spectra of different ferry and small boat noise playbacks recorded with a Brüel & Kjær 8104 hydrophone (s.d. in shaded colours). Note that spectra represent the full passage of each boat normalized peak-to-peak. Spectra settings: sampling frequency, 4 kHz; FFT size, 1024; window type, Hanning; window overlap, 50%.

estuary. Noise level varied from 10 to 40 dB above background (90–100 dB re. 1 µPa calculated in the 0–2000 Hz bandwidth) mostly because the output of the speakers changes depending on tide level (due to changes in water pressure). At tide levels above ∼1.5 m, the variations of playback SPL were under 10 dB. To characterize the playback sounds, we calibrated the recordings using simultaneous measurements with a calibrated hydrophone (Brüel & Kjær 8104, Naerum, Denmark). The boat noise sound files used in the playback were recorded nearby at Air Force Base no. 6 pier. We recorded the noise produced by four small private open deck boats with an outboard engine at 7–20 m from the hydrophone (rms 120–140 dB re. 1 µPa, calculated in the 0–20 kHz bandwidth or rms 104–133 dB re. 1 µPa, calculated in the 0–2 kHz bandwidth), and 8 passages of two ferryboats that regularly cross the Tagus river (30–220 m; rms 122–131 dB re. 1 µPa or rms 117–127 dB re. 1 µPa; background noise ranged from ∼95 to 100 dB re. 1 µPa). Small motorboat sounds used in the playback had a higher spectral variability, whereas the ferries had a lower variability with higher energy up to 450 Hz (Fig. 4B). Two of these sounds were also used on the AEP experiment.

Data processing and statistics
From the recordings, we selected periods in which only two males were vocalizing. This selection and labelling were made with the aid of a Hidden Markov Model automatic recognition system described in a previous paper (Vieira et al., 2015). The labels obtained, indicating the occurrence of BWs and of boat playbacks, were

manually verified and, if needed, corrected. This was particularly necessary during the playback of boat noise when the number of false negatives increased. No discrimination was made between ferries and small private open deck boats. Data labels were imported and analysed with R. Recordings from low tides (water depth below ∼1.5 m) were excluded because of a possible influence on the calling rate as reported by Amorim et al. (2011) and the decrease in playback energy.

To analyse the vocal interactions, each pair of fish was classified according to the distance between them: close distance neighbours (CDN; 2–2.3 m), medium distance neighbours (MDN; 3–4.5 m) and long distance neighbours (LDN; 7–8 m). The phase of the BWs in a fish pair interaction was measured and represented on rose plots, where 360 deg corresponds to the inter-onset interval (i.e. the interval between the beginning of one event and the beginning of the consecutive event) of a pair of consecutive BWs of one fish (see Fig. 5A). These data were obtained for the three interaction distances (CDN, MDN and LDN) both with and without noise playback, in a total of six classes of vocal interactions. Because the inter-onset interval did not show much intra-individual variability, when one fish made more than one BW between consecutive BWs of the reference fish only the first BW was considered. We used the Rayleigh test of circular statistics (Fisher, 1995) to test if the noise playback altered the phase of the BWs between individuals (Vieira et al., 2021).

From 7 days of round-the-clock recordings, ∼4.5 h were selected with only one pair of calling fish. We selected periods where only
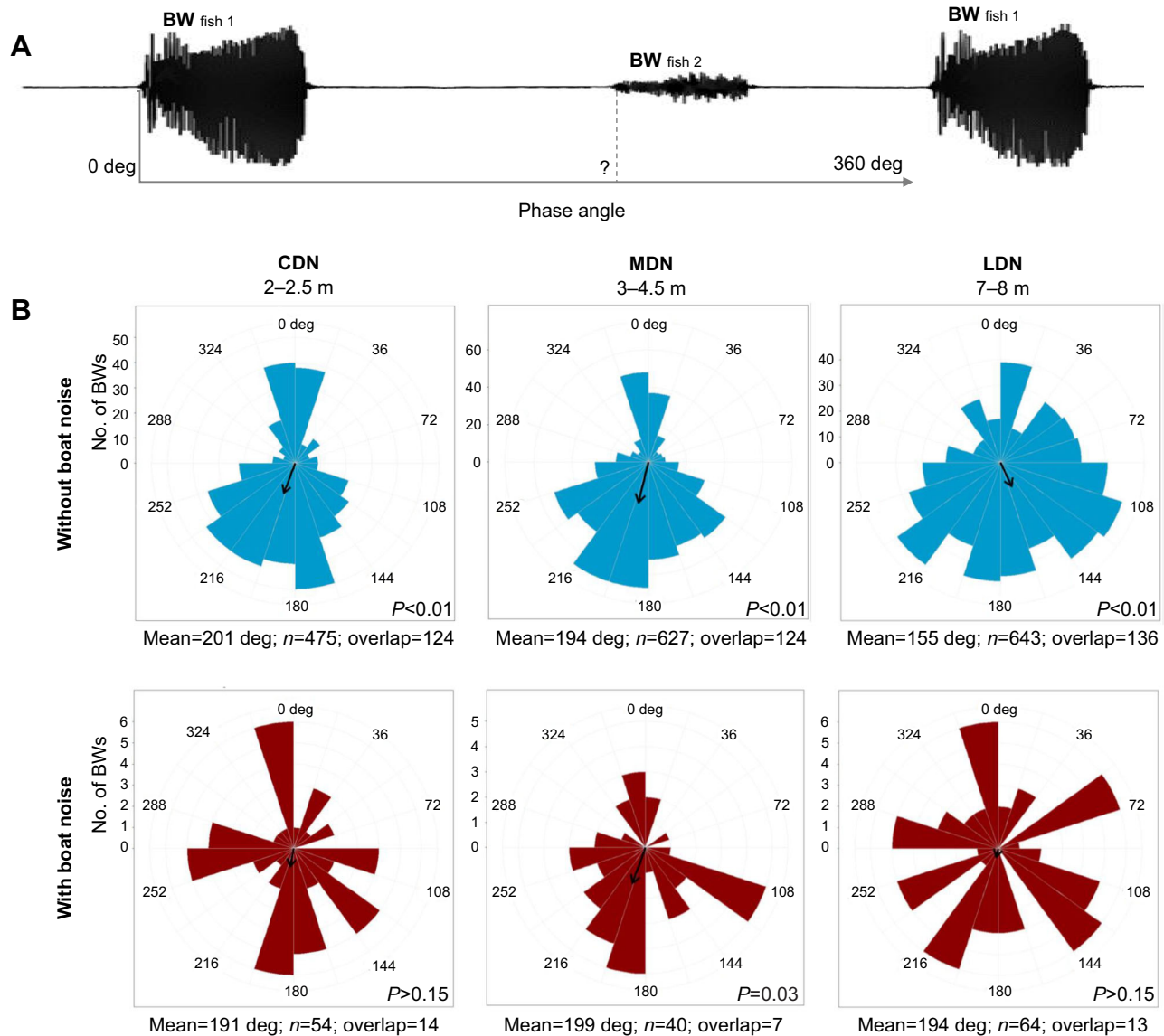
Fig. 5. Relative phase measurement of BW calls of neighbouring Lusitanian toadfish. (A) Scheme of the relative phase measurement. (B) Rose plots showing frequency distributions of relative phases of calls of neighbours. Calls when no boat noise was occurring (top panels) and calls during boat noise exposure (bottom panels) are represented. Pairs of neighbours were separated according to the distance between them: close distance neighbours (CDN), medium distance neighbours (MDN) and large distance neighbours (LDN). Mean vectors are represented by an arrow on each plot. The BWs produced between boat noise playbacks reveal the presence of vocal interactions between fish. In contrast, during the boat noise exposure, the distribution of BWs phase becomes more erratic, pointing to a disruption of the vocal interaction. P-values refer to the Rayleigh's uniformity test; n, number of BWs; overlap, number of BWs that at least partially overlapped.

two males were vocalizing to ensure that putative interactions in the vocalizations of a pair of males were not disturbed by the vocal activity of another male in the vicinity. A total of 11 males were considered in 13 pairwise interactions. While we have not conducted an individual identification of males, the monitoring of continual vocal activity strongly suggests that only one male was recorded in each nest. Note that there is a high degree of stereotypy in the BWs of one male as reported by Amorim et al. (2011), and that one breeding male typically stays and defends its nest from intruders while providing parental care (Almada and Faria, 2004; Amorim et al., 2010a; Vasconcelos et al., 2010). Altogether, we considered 1 h 19 min with four pairs of fish separated by 2–2.3 m (CDN); 2 h 9 min with three pairs of fish at 3–4.5 m (MDN); and 1 h 12 min with four pairs of fish at 7–8 m (LDN). In these three

subsets of the recordings, we detected a total of 1650 BWs produced during CDN interactions, 1871 BWs produced in MDN interactions and 1581 BWs produced in LDN interactions. From these data, the phase of 1903 pair interactions was obtained from 5102 labelled BWs. The boat noise playbacks (n=106) throughout the analysed recordings had a SPL of 124.1±4.3 dB re. 1 µPa (mean±s.d.). The sounds were recorded at the mid-lateral position of each nest (distance to speaker 1–2.24 m).

To investigate the effect of the boat noise playback on calling rate, calculated with the inter-onset interval (Fig. 6A; Ravignani and Norton, 2017), we performed a generalized linear mixed model (GLMM) analysis. We used the flexible, penalized, quasi-likelihood method (family-Gaussian; link-log) that is suitable for over-dispersed data, crossed random effects and unbalanced design.
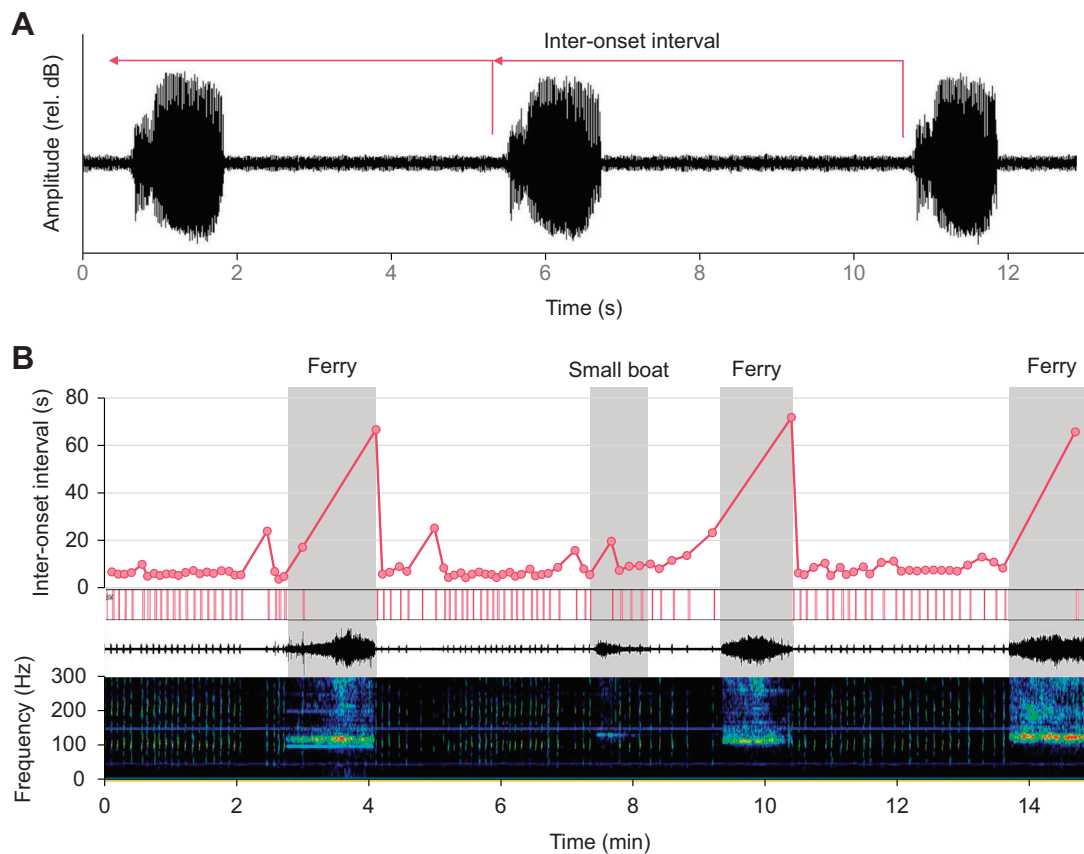
Fig. 6. Example of a monitored fish vocal activity during the playback experiment. (A) Schematic figure of inter-onset intervals. (B) Variation of the inter-onset intervals through time of an example recorded on hydrophone 6; including labels for BWs (red vertical lines) and playback noise (grey). Bottom panels represent the spectrogram and oscillogram; spectrogram settings: sampling frequency, 4 kHz; FFT size, 1024; window points, 1000; window type, Hanning; window overlap, 50%. Note that the loudspeakers used in the field playbacks have a poor performance and lose power at frequencies below 100 Hz.

Individual males (n=11) were included as a random factor in the model. This analysis was performed using the previously 5102 labelled BWs of fish calling in pairs.

RESULTS

Boatwhistle representation in the auditory evoked potentials

In this work we chose two of the BWs used in Alves et al. (2016) to test the effect of boat noise in impairing the toadfish capabilities for discriminating conspecific advertisement sounds. Table 1 shows the mean values of the estimated active space of the Lusitanian toadfish BWs in the presence of boat noise, considering the propagation of the BWs in a shallow (max ~2 m water height) breeding site. Different boat noises affected the active space differently (Kruskal–Wallis, χ^2_5=25.2, P=0.0001).

The small outboard motorboat noise used in our experiment, with its strong low frequency sound components, significantly reduced the estimated active space of both BWs when compared with no noise conditions (10.4 m to 2.5 m, P<0.001, for BW2; 6.4 m to 2.0 m, P=0.03, for BW1; Dunn test). Regarding the AEP response to BW2 (the most severely affected) it is noticeable that the number of pulses present in the responses at 5 and 7.5 m is reduced in relation to the AEP responses measured without boat noise (Fig. 7). The AEP response to BW1 shows a similar pattern to that for BW2, with no significant differences in the estimated active space of BW1 and BW2 (P=0.8). A spectrum analysis of the AEP response to BW2 also reveals that, while the no-noise condition shows a gradual decrease of the energy with increasing distance, in the presence of

the outboard motorboat noise the energy reaches the lowest values at shorter distances, above 2.5 m (Fig. 8), contrasting with the response to the BW+ferry condition. The difference is particularly clear in the 50–200 Hz range. These frequencies are likely important in this species' communication, as they encompass the response to the dominant frequencies of the BWs (120 Hz, double the ~60 Hz fundamental frequency of BW2; Fig. 2). The small outboard motorboat noise also influenced fish hearing variability as the standard deviation of the hearing distance estimations were lower when compared with the no-noise condition (Table 1).

The ferry noise appears to have a smaller impact on the AEP representation of BWs, either reducing the active space less than the outboard motorboat (10.4 m to 6.3 m for BW2; P=0.096) or apparently not affecting it at all (6.4 m to 6.7 m for BW1; P=0.97; Table 1). However, no significant differences were observed in the estimated active space of both BWs under ferry noise conditions (P=0.9). The AEP responses to BW2 were more similar between the BW2 and BW2+ferry stimulation than with the BW2+small boat, as revealed by the response pattern (number of identifiable pulses) at 5 and 7.5 m (cf. Fig. 7). The AEP analysis of BW1 showed no relevant difference between ferry noise and the no-noise condition. The spectra of the AEP responses to BW2+ferry revealed a gradual decrease of energy at the most relevant hearing frequencies, similar to the no-noise condition and contrasting with the BW2+small boat spectra (Fig. 8). The ferryboat noise increased the variability of fish hearing distance estimations with the standard deviation increasing comparatively to the no-noise condition.

Table 1. Estimated mean maximum distance±s.d. at which the fish can correctly encode the amplitude modulations of the stimulus BW in the presence or absence of anthropogenic noise

	No noise*		Small boat		Ferry	
	Fish ID	Detection distance (m)	Fish ID	Detection distance (m)	Fish ID	Detection distance (m)
+BW1	2	10	32	1	20	10
	3	7.5	33	2.5	21	7.5
	5	5	34	1	22	10
	8	5	35	2.5	23	5
	9	7.5	36	2.5	24	5
	11	5	37	2.5	25	2.5
	13	5				
Mean±s.d.		6.4±2.0		2.0±0.8		6.7±3.0
+BW2	1	12.5	14	2.5	26	7.5
	4	7.5	15	2.5	27	10
	6	10	16	2.5	28	7.5
	9	10	17	2.5	29	7.5
	10	12.5	18	2.5	30	2.5
	12	10	19	2.5	31	2.5
Mean±s.d.		10.4±1.9		2.5±0.0		6.3±3.1

*From Alves et al. (2016).

Fish vocal behaviour patterns

In most cases, we observed alternation of BWs from the fish pair with a phase between 155 deg and 201 deg (Fig. 5). Boatwhistle overlap occurred in 15–26% of cases. A Rayleigh test of uniformity with unspecified mean direction was performed on the six classes considered (3 interaction distances×noise/no noise playback). The phase of BWs produced during no boat noise playback has a clear non-uniform distribution (CDN: $z=0.25$, $P<0.001$; MDN: $z=0.33$, $P<0.001$; LDN: $z=0.21$, $P<0.001$). A Rayleigh test with specified mean ($\mu=180$ deg) further suggested a non-uniform distribution consistent with alternation of BWs between fish at close and middle distances ($z=0.21$, $P<0.001$; $z=0.26$, $P<0.001$), but not at longer distances ($z=0.04$, $P=0.06$). In contrast, during exposure to boat noise playback, the distribution phase of BWs is more variable. The phase of BWs is not significantly different from a uniform distribution for close and longer distances (CDN: $z=0.15$, $P=0.3$; LDN: $z=0.07$, $P=0.7$), but still significant for middle distances (MDN: $z=0.30$, $P=0.03$).

During boat noise exposure the inter-onset interval of BWs increased (GLMM: $n=5041$, $\beta=-0.34$, s.e.m.$=0.06$, $t=-5.02$, $P<0.001$), corresponding to a decrease in the BW calling rate. Fig. 6 shows an example of a clear increase in the BW inter-onset interval during boat noise playback.

DISCUSSION

There are a growing number of studies on the negative effects of noise on aquatic organisms, ranging from lowering attack rate of carnivorous fish (Purser and Radford, 2011; Hanache et al., 2020), decreasing anti-predator behaviour (Simpson et al., 2016), altering movement patterns (Becker et al., 2013; Sarà et al., 2007) and social behaviour (Bruintjes and Radford, 2013; Sebastianutto et al., 2011), and impacting spawning (de Jong et al., 2018). However, not only are studies on the effects of boat noise on acoustic communication sparse, but evaluations of its effect are also made under different paradigms and protocols, making comparisons difficult. Understanding how boat sounds and other types of anthropogenic noise affect communication in fish, and thereby their fitness, is a pressing matter (Brumm, 2014). In this regard, estimation of communication active space should be based on the perception of the information content and not just energy detection of signals

since sound characteristics could be relevant in social interactions including mate choice (Amorim et al., 2015).

In previous studies, the active space of the Lusitanian toadfish (Alves et al., 2016), a vocal teleost fish that depends on acoustic communication for successful reproduction (Vasconcelos et al., 2012; Amorim et al., 2016), was evaluated and the vocal patterns of toadfish in choruses was described (Jordão et al., 2012; Vieira et al., 2021). The estimated range varied between 6 m and 13 m in a shallow breeding area (Alves et al., 2016). The average range for BW1 was 6.4 m and for BW2 was 10.4 m (Table 1). While chorusing, males avoid overlapping their calls with their neighbours, leading to a pattern of antiphony alternation (Vieira et al., 2021). We assessed the impact that boat noise causes on this species' active space and vocal behaviour. To achieve these goals, we: (1) measured the reduction in the maximum distance the BW is represented in the AEP after embedding it in boat noise and (2) acoustically monitored breeding males exposed to boat noise playbacks in the Tagus estuary to assess vocal interactions. Our results indicate that boat noise lowers communication distance, interferes with male–male vocal interactions and provokes a significant decrease in the males' calling rate during boat noise playback. In this species, females are attracted to the breeding sites and to the nests by advertisement BWs (Amorim and Vasconcelos, 2008) and this vocalization is also used as a territorial 'keep-out' signal (Vasconcelos et al., 2010). The BW pulse period, amplitude modulation and calling rate are correlated with male quality (Amorim et al., 2010b) and these acoustic signals have potential for individual recognition (Amorim and Vasconcelos, 2008; Vieira et al., 2015). These effects combined will likely imply fitness costs.

Boatwhistle representation in auditory evoked potentials

We evaluated how noise recorded from two different boats, a ferry and a small fishing boat with an outboard engine, impacted the communication range of BWs. As expected, our results suggest that boat noise impacts communication range depending on noise spectral content, besides noise level. While the fishing boat noise, with more energy at the BW spectral range, had a severe impact on communication range (~75% reduction), the ferry noise had a smaller impact owing to its lower energy content in the BW frequency range, thus causing less masking. Note, however, that the spectral
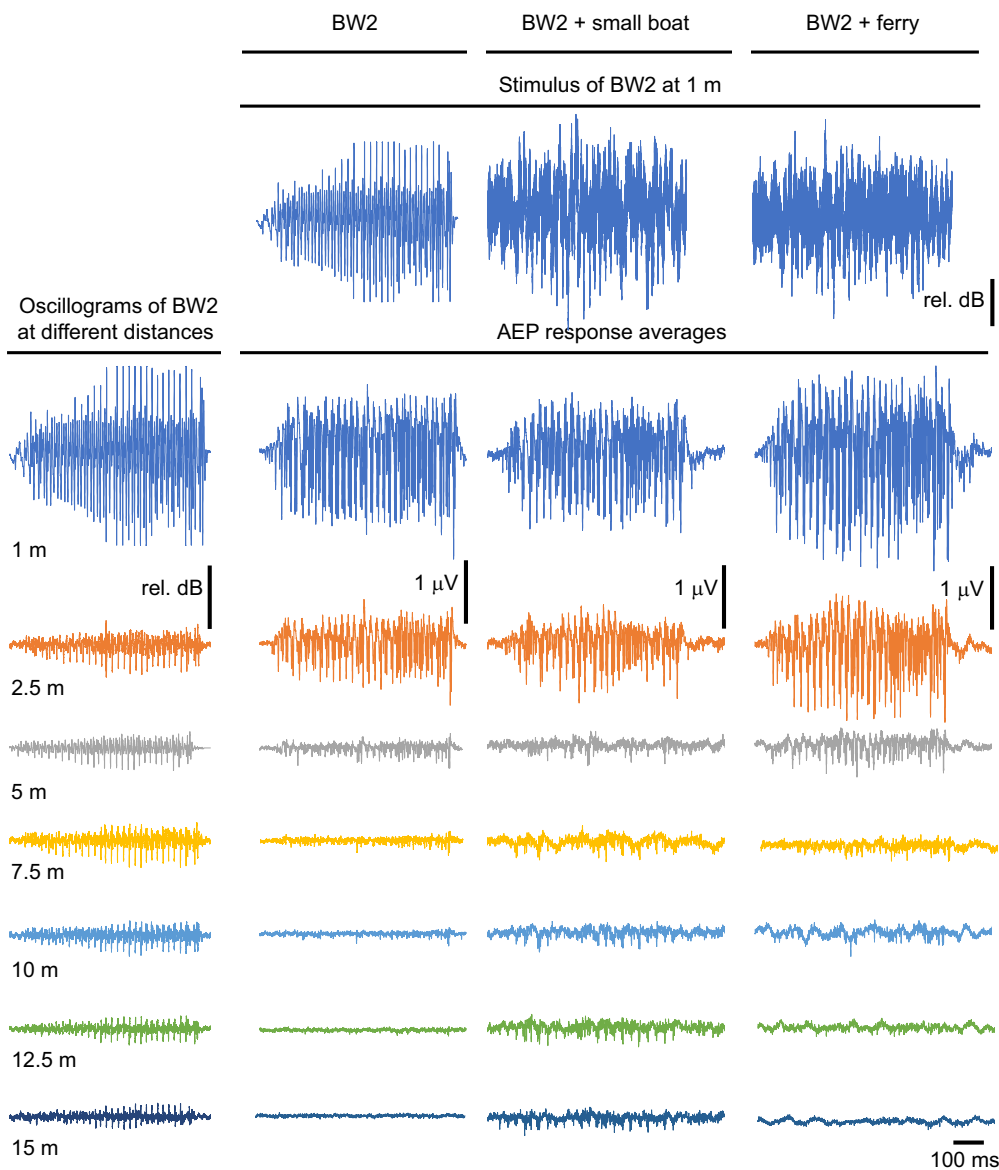
Fig. 7. Oscillograms and AEP response averages to BW2 alone, or BW2 mixed with sounds of a small boat or ferry. Oscillograms of BW2 recorded at different distances (left) and AEP response averages to BW2 without noise (fish no. 9; from Alves et al., 2016), BW2 mixed with small outboard motorboat noise (fish no. 18) or BW2 with ferry boat noise (fish no. 29) (right). The playback of the BW2 recorded at 0.1 m was reproduced with an average root mean square (RMS) SPL of 130 dB (re. 1 μPa), corresponding to a fish recorded at 1 m. At the top are stimuli of the BW2 alone or mixed with small motorboat or ferry noise.

content of different boats/engines is variable. For example, Sarà and collaborators (2007) recorded noise from ferries that had more energy at lower frequencies than that exhibited by small boats with outboard motors. The masking effect is complex since it influenced both the temporal patterns (the number of distinguishable pulses) as well as the frequency content of the AEP response to the BW.

In the absence of boat noise, the BWs can be detected up to a distance of 6–13 m, dependent on their spectra, as reported by Alves et al. (2016). This difference is related to the spectral content of the distance-attenuated BW above background noise that exceeds the hearing threshold of the species. This is in accordance with what would be expected by the power spectrum model of masking (Dooling et al., 2015). Nevertheless, boat noise masking resulted in similar active spaces of the two BWs tested, despite their spectral differences recorded at close range. Several aspects, such as stronger attenuation at lower frequency components in this shallow habitat (Mann, 2006) might contribute to these results (Fig. 2).

Hearing threshold estimations using the AEP technique yield consistently higher values than those produced by other physiological (such as saccular potentials) and psychophysical methods (Sisneros et al., 2016; reviewed in Ladich and Fay, 2013) and therefore are likely to underestimate actual distances for effective communication. Nevertheless, we believe that our comparative assessment of reduction of communication active space might be useful since it is based on the same AEP methods. Furthermore, by showing that the pattern of the envelope of the AEP correctly followed the pattern of the envelope of the stimulus, we can infer that information relevant for social interactions is available at the brain level.

This work can yield important insight on the severity of the impact of anthropogenic noise on acoustic communication. Nevertheless, full understanding of the active space of an acoustic signal, based on recognition of the information present in the sound, can only be addressed with behavioural experiments designed specifically for this purpose. Furthermore, note that the AEP setup may not allow for the best replication of the particle motion of sounds in the field, because of the proximity of the speaker and the singular direction of particle motion, which could influence hearing (Popper and Hawkins, 2018). We additionally recorded some boats in the same location using both an accelerometer and the Brüel & Kjær 8104 hydrophone (examples of the passage of a ferryboat are
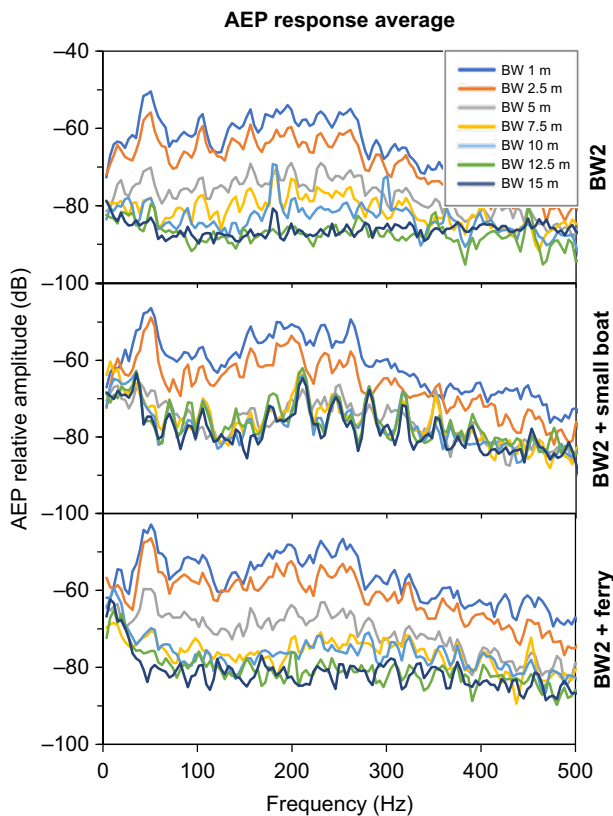
Fig. 8. Power spectra of AEP responses to BW2 alone, or BW2 mixed with sounds of a small boat or ferry. BW2 without noise (fish no. 9; top), BW2 mixed with small outboard motorboat noise (fish no. 18; middle) or BW2 with ferry noise (fish no. 29; bottom) with the different distances identified. The responses utilized to calculate the spectrograms are those presented in Fig. 3.

shown in Figs S1 and S2). From these recordings, it is obvious that the relative position of the fish and the passing boat matters. Furthermore, we observed overall comparable acceleration on all 3 axes in the moment the boat passes by the accelerometer (between 50 and 100 m away in several passages). In the tank, the particle motion is dominated by the vertical axis. Further studies are needed to understand masking in real conditions.

Fish vocal behaviour patterns

Our results confirm that the temporal patterns of the Lusitanian toadfish call can change in the presence of boat noise, usually increasing the inter-onset interval during boat noise playback. This is consistent with the effect observed in the oyster toadfish (*O. tau*; Krahforst et al., 2016; Luczkovich et al., 2016). Radford and collaborators (2014) described some possible coping mechanisms that signallers could adopt in the presence of anthropogenic noise including noise avoidance, changes in temporal parameters, amplitude increase (i.e. the Lombard effect; Holt and Johnston, 2014; Zollinger and Brumm, 2015), frequency shifts and changes in signalling modality. The Lusitanian toadfish appears to lower calling rate (as evaluated by the inter-onset interval) in the presence of boat noise, which may configure a noise avoidance response.

Previously, we observed that in Lusitanian toadfish, close neighbours tend to sing with a phase offset near 180 deg (antiphony alternation), but this interaction pattern fades at larger distances (Vieira et al., 2021). In this study, we monitored the impact of boat noise playback in vocal interactions between

breeding males in their natural habitat. Our results indicate that boat noise interferes with the fish interactions, turning the usual alternation into a random vocal pattern, suggesting that male interaction is hampered. Signal timing and male–male interactions can play a crucial role in animal communication (Bowling et al., 2013; Ravignani and Norton, 2017). In normal circumstances, vocal alternation between males could increase the chances to attract females. This may also assist in the formation of groups of males (by congregation and/or spacing), as suggested for other animals (Alexander, 1960; Fish, 1972). For the Lusitanian toadfish, we still do not fully understand the importance of the male–male vocal interactions, but there is an active adjustment of calling rates of each male to the neighbours' vocal activity (Jordão et al., 2012; Vieira et al., 2021). Other fine scale interactions occur in vocal patterns in fish. For example, in *O. tau*, a species from the same family as the Lusitanian toadfish, males produce grunts simultaneously with other males' boatwhistles, to interfere with their detectability (Mensinger, 2014). Further studies should address in detail the consequences of a disruption in the male–male vocal interactions.

The observed change in the phase of the calls from a unimodal distribution into a uniform distribution is likely caused by masking, leading to a reduction in the toadfish communication range. This is consistent with the results from our AEP experiments. However, multiple factors could explain the shifts observed. For example, anthropogenic noise may negatively affect receivers by distracting them and preventing them from interacting in the most advantageous manner. Some evidence exists that fish are distracted by anthropogenic noise (Purser and Radford, 2011; Voellmy et al., 2014). Furthermore, boat noise can also increase stress levels that can suppress reproductive behaviour, including acoustic signalling (Cox et al., 2018). On the other hand, these fine-scale consequences may translate into a decrease in parental care. Picciulin and colleagues have observed a reduction in parental care in the pomacentrid *Chromis chromis* in the presence of boat noise (Picciulin et al., 2010). In the spiny chromis (*Acanthochromis polyacanthus*), motorboat noise playback affected parental behaviour and significantly reduced the offspring survival rate (Nedelec et al., 2017). Additionally, individuals of *Neolamprologus pulcher* exhibited a context-dependent behavioural shift in the presence of boat noise (Bruintjes and Radford, 2013). A broader analysis considering the effects of noise on large-scale behavioural temporal patterns and accounting for the reproductive success would allow a better understanding of the effects of this stressor on the Lusitanian toadfish.

Conclusions

Overall, the active space of the Lusitanian toadfish boatwhistle as estimated with AEPs varies between 6 and 10 m, but boat noise may reduce this range by ~75%, depending on spectral content of the noise. In the natural habitat, boat noise exposure decreases male calling rate and causes the vocal interactions between males to lose their patterns. Future studies should look in more detail into possible coping communication mechanisms that these animals may use in the presence of this widespread stressor, as well as evaluating direct fitness impacts.

Competing interests
The authors declare no competing or financial interests.

Data availability

Custom-written software (P.J.F. and M.V.) for signal averaging is available on request from the authors.

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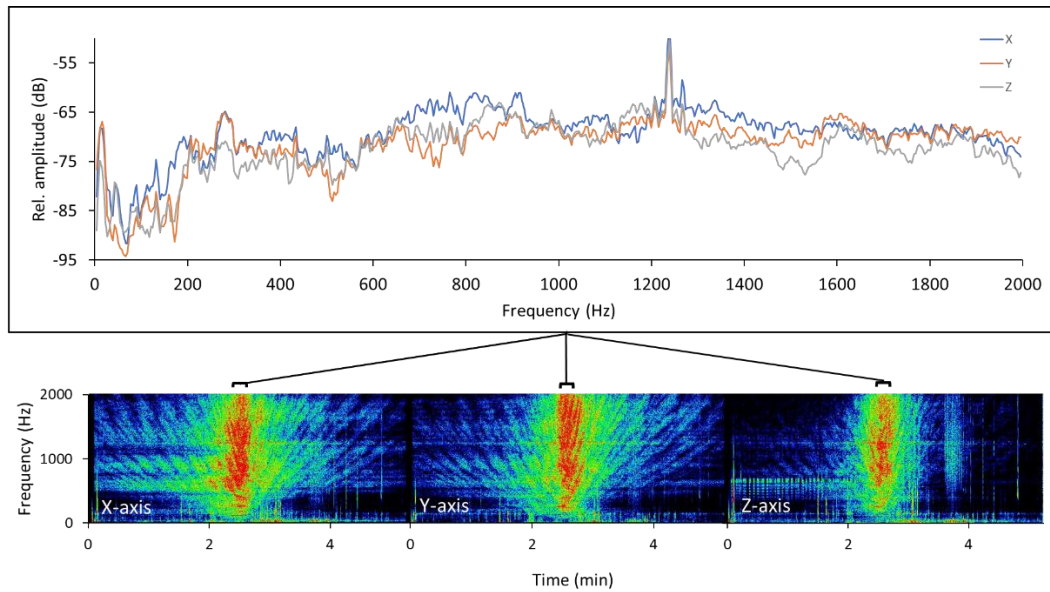
Fig. S1. Spectrograms of a ferryboat passage recorded with a 3-axes accelerometer (x,y and z axis) and power spectra of 3 seconds at the point of closest distance to the recording device. Spectra settings: sampling frequency, 4 kHz; FFT size, 1024; Hanning; window overlap, 50%.
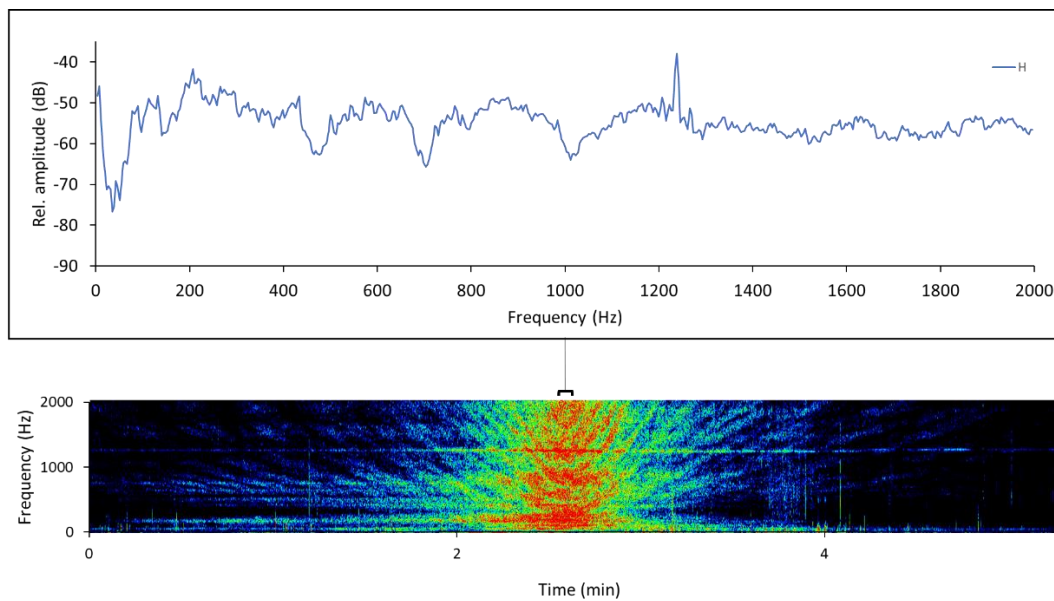

Fig. S2. Spectrograms of a ferryboat passage recorded with a hydrophone (B&K 8104) and power spectra of 3 seconds at the point of closest distance to the recording device. Spectra settings: sampling frequency, 4 kHz; FFT size, 1024; Hanning; window overlap, 50%.